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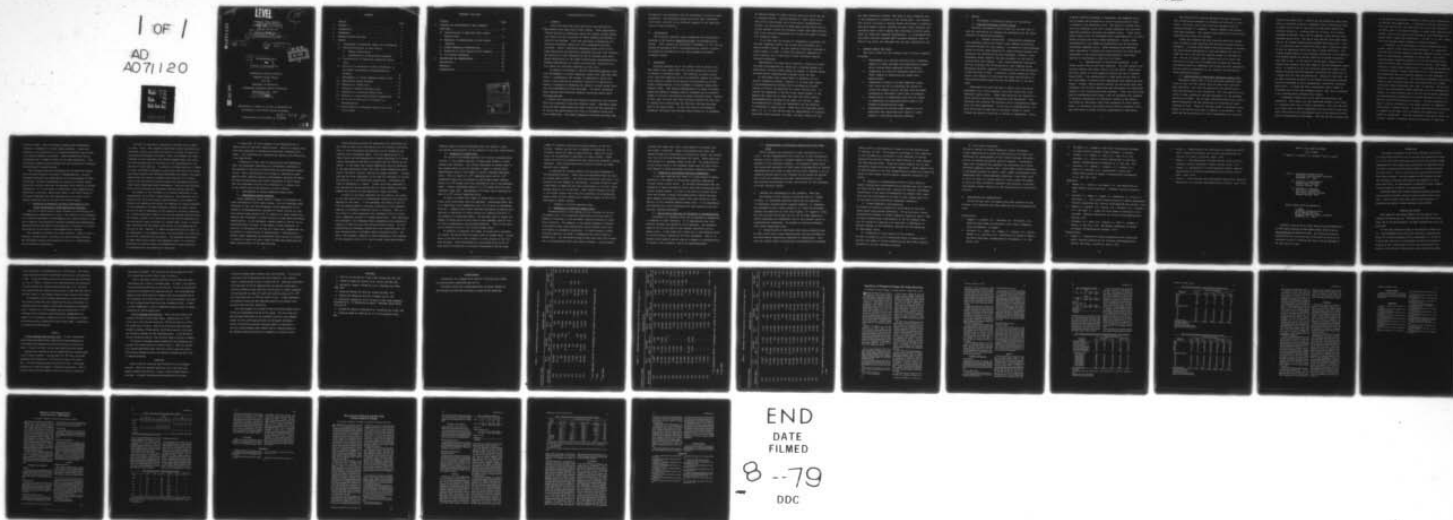
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## Histocompatibility Typing

### A. ABSTRACT:

During the past year major qualitative and quantitative improvements have been made in HLA-D typing. These improvements include advances in homozygous typing cell typing, primed lymphocyte typing and the utilization of these reagents and methods in parallel test systems. First the efficiency of performing and analyzing homozygous typing cell (HTC) experiments has been increased. Second, additional homozygous typing cells of both known specificity and previously undefined type have been obtained. The selection of this laboratory as one of fourteen major HLA-D typing laboratories by the Eighth International Histocompatibility Workshop demonstrates our current international recognition and will assure our continued leadership in this area.

In addition to progress in the HTC methodology, several important developments in primed lymphocyte typing (PLT) have been made. First, a fundamentally new concept in experimental design will permit a more precise and consistent definition of specificity. Second, a technique of expanding the number of primed typing cells over one hundredfold has been developed. And third, procedures which identify and physically separate functional memory cells have been devised.

Finally the techniques and reagents developed by this contract have led to advances in parallel research areas. Homozygous typing cells and HLA-D typed panel were used to determine the association of certain HLA specificities and level of peripheral blood cytotoxic lymphocytes. The primed lymphocytes technique has been used

to identify a new potential role for interferon in clinical transplantation. And homozygous typing cells have been transformed into B-cell lines for use as standard reagents in the identification of B-cell antisera.

#### B. INTRODUCTION

The following technical report summarizes the second year's progress on ONR contract N000-14-77-C-0747 "Histocompatibility Typing". This contract was initiated on September 15, 1977 for the purpose of developing HLA-D histocompatibility testing to permit the potential availability of non family member bone marrow transplant donors.

#### C. BACKGROUND

Military personnel are at far greater risk of contact with bone marrow toxic agents than the civilian population by virtue of working in hazardous areas. The necessity of using various toxic fuels and chemicals as well as potential exposure to irradiation produce a hazard which requires the availability of bone marrow transplantation in the treatment of aplastic anemia from whatever cause. Bone marrow transplantation in cases of aplastic anemia has become an accepted form of treatment and is currently funded in civilian institutions by health insurance plans. However, at this time, the only long term survivors treated with this form of therapy have occurred when HLA identical siblings have been used as the marrow donor. As only one third of the potential transplant recipients have matched sibling donors it is necessary

to identify methods of tissue matching which will allow the use of unrelated donors. Improved methods of tissue typing would then allow a much broader use of bone marrow transplantation. Further, the identification of those specificities which must be closely matched in donor and recipient verses those specificities with little clinical consequence will broaden the number of potential transplant donors.

It is clear that the success of transplants is greatest with the most compatible grafts, and is much less successful with poorly matched tissues. We have therefore initiated a program of tissue typing, in order to define the essential components of graft rejection and to seek ways to alter the body's natural rejection mechanisms.

Approximately two years ago we received a contract to study the human histocompatibility system and to develop methods for large-scale, accurate tissue typing for support of ongoing kidney and bone marrow transplantation programs within the Navy.

At the present time tissue typing is essential for all current transplant programs. However, it is clear that typing of histocompatibility antigens must be extended and further refined to fulfill its potential as a major clinical tool. To outline the complexity of the HLA tissue typing system, it now appears that there are at least five gene loci which control histocompatibility antigens on the human cell surface. These histocompatibility antigens are responsible for the rejection or acceptance of tissue grafts. These five loci have been called HLA-A, -B, -C, -D, and -DR and code for approximately 75 currently identified unique antigens. Of these, the HLA-D region loci are

the least understood; however, they seem to play a dominant role in the acceptance or rejection of the tissue graft. The research that we have initiated is directed toward the problem of examining this HLA-D genetic region, defining the HLA-D locus antigens, and cataloging them in such a manner that individuals could be easily typed for these most important determinants. It is estimated that the eleven currently accepted HLA-D antigens represent about one third of the total, the remainder have not been identified as yet.

D. RESEARCH DESIGN AND PLANS:

The initial plans for this contract were to follow a sequence of events.

1. Establishment of a contract facility with a laboratory capacity to study the human histocompatibility system and do routine mixed lymphocyte culture testing.
2. Preparation of a panel of homozygous typing cells (HTCs) capable of identifying the common HLA-D specificities.
3. Preparation of panels of primed lymphocyte typing (PLT) cells for use in confirming the results of initial HTC typing, and for use when rapid knowledge concerning an individual's HLA-D type is essential.
4. Identification of alloantisera that will be capable of identifying the associated HLA-DR types in the standard microcytotoxicity assay.
5. Cryopreserving and storing these reagents in such quantities that they would allow typing of large numbers of individuals whenever necessary.



## E. RESULTS

### 1. Development of Homozygous Typing Cell Methodology

#### 1.1 Improved Efficiency of HLA-D Typing

Extensive improvements have been made over the past year in both the organization of reagents and experiments and the implementation of specialized equipment.

a. Reagent organization: Cryopreserved lymphocytes from homozygous typing cell donors, panel members and families have been obtained in large numbers. An extensive cross-referenced system of storing these materials in liquid nitrogen freezers and organizing the information has been developed. Further, a data management system (AIMS-02) has been purchased for use with an in-house Wang 2200 computer. Data, cross-referenced information, and cell inventory will be maintained in this computer system permitting efficient updating of inventories and cross-referencing of specificities on hand. In addition to data storage and referencing, this system will facilitate the rapid identification of relationships between specificities and definition of new HLA-D types.

Experiments have been designed to permit very large matrix experiments (32 or more responder cells each tested with 30-60 homozygous typing cells). These large matrices are required to obtain accurate statistical information from experiments. This laboratory has pioneered the exclusive use of cryopreserved lymphocytes for these experiments. By the use of cryopreservation, all cells which must be typed can be collected, stored in liquid nitrogen and quality controlled in advance of experiments. Using



a second technique pioneered at Georgetown, the responder cells can be thawed and distributed on tissue culture plates 24 hours in advance of the stimulator cells. The ability to handle stimulator and responder cells from a single experiment over two days simplifies technical protocols and reduces errors. Third, homozygous typing cells are irradiated after cryopreservation (at  $-80^{\circ}\text{C}$ ) saving critical time during large experiments. Fourth, these irradiated HTC's are thawed and distributed onto the previously distributed responder cells. These procedures have permitted three individuals to perform the tissue cultures for large matrix experiments in two normal working days whereas it previously required 10 individuals to work for 16-20 hours.

b. Implementation of Specialized Equipment: A new tissue culture facility has been completed. The room contains four verticle laminar flow hoods, each of six foot width. These hoods have been placed on custom supports allowing the use of standard height chairs. The configuration of these hoods allows three or more individuals to perform large experiments with reduced physical strain. Further, easily movable cabinets and partitions were used throughout the facility, permitting easy maintenance and simplified changes in room configuration when necessary. Other room facilities such as refrigerated centrifuges, sink and incubators are arranged to minimize interference of individuals working in the laminar flow hoods by individuals using other equipment for their experiments. On the whole, the tissue culture facility optimizes clean, open work space to permit efficient performance of experiments.

New scintillation counting equipment has been installed. This scintillation equipment is reliable and efficient in operation. Currently, data output from this equipment must be manually entered in a computer. However cassette tape outputs have been purchased. The cassette tape system will allow greatly simplified data entry and analysis.

A Wang 2200 computer has been installed which will accept data on magnetic tape cassettes from the scintillation counting equipment. A program which permits this Wang computer to analyze the large matrix experiments is under development and will be ready for use at the time the cassette system is available for use. The use of a small local computer will be of great time savings as the data can be automatically entered and analyzed, the results saved for comparison with future and past experiments and the results incorporated in the inventory and specificity cross-referencing system.

#### 1.2 Identification of Additional Homozygous Typing Cells

Homozygous typing cells of both previously undefined specificity as well as other typing cells of known HLA-D type have been identified. One rare new specificity was defined using a technique developed in this laboratory. This new specificity was found in three individuals who were homozygous for the serotype specificity HLA-B40. Two of these individuals were from a family where homozygosity had been defined by an intrafamilial mixed leukocyte culture. These two siblings failed to stimulate either parent in an MLR but were able to respond to the parents. This permitted the identification of HLA-D homozygosity but could not

identify the specificity. Specificity was defined by using these cells to stimulate a panel of responder cells of known HLA-D type. As these cells failed to type cells of known HLA-D specificity (HLA-Dw1 through 11), a second panel of 30 cells, all of which serotyped as HLA-B40, were used as responder cells against these proposed typing cells. The use of many HLA-B40 cells takes advantage of the linkage disequilibrium between HLA-B and HLA-D. That is, an individual with a particular HLA-B type is much more likely to have an associated HLA-D type than a random individual. This linkage disequilibrium is most strongly pointed out in the association between HLA-B8 and HLA-Dw3 where 60% of HLA-B8 individuals also type as Dw3. In the case of the B40 panel, three of the twenty seven possible cells were typed by the new homozygous typing cells. In addition to the two initially identified typing cells, another unrelated HLA-B40 homozygous individual was identified as a typing cell for this new specificity. Because of this work, two of these cells were accepted in the Eighth International Histocompatibility Workshop as cells representing a previously undefined specificity. If these new HTC's consistently type a number of unrelated cells during the workshop testing, they will be assigned a new internationally accepted HLA-D number.

A homozygous typing cell of the rare Dw9 specificity was identified. This typing cell was identified because it was found in a platelet donor who is HLA-B14 homozygous. This cell was used as a typing cell in several experiments and was found to type only cells identified as Dw9 heterozygous cells in the Seventh International Histocompatibility Workshop. This new Dw9 HTC is being used

in the Eighth International Workshop to permit other laboratories to identify this specificity. In addition to the new specificity HTC's and the Dw9 HTC, two additional HTC's from this laboratory are being used in the Eighth workshop to define known specificities. Further, many of the typing cells defined by this laboratory are widely used by other HLA laboratories. Our DW1, 2, 3, and 4 typing cells have become widely accepted as standard reagents.

In addition to the improvement in typing reagents and techniques, several other areas are being advanced. In cooperation with the Naval Medical Research Institute Biometrics group, refinements in data analysis techniques are being made. The current internationally accepted technique is an ad hoc procedure which does not produce firm, statistically valid results. The current technique, called double normalization, is performed by determining a relative capacity of each stimulator to act (75th percentile of the stimulation cpm) and relative capacity for each responder to replicate (75th percentile). Each stimulator-responder experimental value is then corrected using the 75th percentiles and a number called the double normalized value (DNV) is obtained. Values less than 40 are considered as typing responses, 40-60 as indeterminate values, and values greater than 60 are called non-typing responses. The approach being developed with the Navy Biometrics group is to define a value with statistical validity. The basic approach has been to utilize information on the known kinetics of lymphocyte replicating in tissue culture. As these replicating cells increase in numbers by dividing, that is doubling and redoubling, their growth is logarithmic. Thus the logarithm of the cpm is first calculated. Second, a weighted average and variance of replicate



values is taken. Then the average response and corresponding variance is compared to a known typing response. Using this technique, the probability of a responder-stimulator pair producing a typing response is defined. These probabilities can be accumulated for a number of tests of the same specificity. This statistical approach should produce far more accurate and unbiased identification of specificities.

Over the past year this laboratory has collaborated closely with the Duke University Division of Immunogenetics. We have performed numerous joint experiments on the relationship of HLA-D and HLA-DR. We have concentrated our efforts on cellular HLA-D typing and the Duke group has concentrated their efforts on HLA-DR serotyping. In a joint paper in preparation, data supporting the hypothesis of multiple, duplicated, genes in the HLA-D region of the sixth chromosome has been hypothesized as the cause of the complex reactions seen in the HLA-D and HLA-DR typing.

### 1.3 Selection of Laboratory as Major Contributor to the Eighth International Histocompatibility Workshop

Each two to three years a major congress of contributing histocompatibility laboratories is held. The purpose of this workshop is to advance knowledge of histocompatibility identification in man and to develop internationally accepted standards and nomenclature. Laboratories are selected for participation on the merit of current contribution to the field of histocompatibility testing.

The workshop is divided into three sections: HLA-A,-B and -C serology, HLA-DR (B-cell) serology, and HLA-D cellular testing. The Georgetown facility was selected as one of 14 international HLA-D reference laboratories.



The work of each HLA-D laboratory is divided into a number of tasks. First, test reagents identified by each laboratory are collected, quality controlled and sent to a central location for distribution throughout the world. Next, the reagents are redistributed to each laboratory for testing against local test cells. For this workshop most of the work is to be concentrated in the testing of large families. In addition to these families, a number of local individuals selected to carry rare types are to be tested. Once these families and selected individuals are tested, the experimental data are coded on standard forms and sent to a central facility for statistical analysis along with serotyping data and other sixth chromosome marker information. Data are then analyzed both in each contributing laboratory and the central facility.

The design of this workshop is of crucial importance to the understanding of the HLA-D region genetics. The extensive use of families will allow the precise identification of the number of genetic loci producing the results we interpret as typing responses in the performance of mixed leukocyte cultures for HLA-D typing. We will be able to determine if the results we currently interpret as a single specificity might in fact be two separate specificities. For example, should it be found that the Dw5 and Dw6 specificities are alternative forms (alleles) of a single gene (locus), a chromosome inherited from one parent could result in the expression of only Dw5 or Dw6. However, if these two specificities are found to be represented on two separate genes, one could expect to find both Dw5 and Dw6 inherited from one parent. The physical relationship of these genes which control HLA expression then, is of crucial importance in defining those histocompatibility types important to transplantation and immunologic regulation.

A second part of this workshop is the identification of typing cells for new HLA-D specificities. Thirty six typing cells have been submitted as representing previously undefined HLA-D types. This laboratory has submitted two typing cells identifying a new specificity.

In addition to this input of typing cells and experimental data, the Georgetown laboratory will extensively contribute to the analysis of the data collected from all participating laboratories. Major contributions will be made in identifying known specificities, defining genetic relationships, defining new specificities and developing the overall principles of HLA-D typing. Thus the Georgetown facility will contribute in a central role in the current and future direction of HLA-D testing.

## 2. Advancements in Primed Lymphocyte Typing (PLT)

### 2.1 Experimental Design Concepts

Primed lymphocyte typing offers a number of advantages over other techniques in HLA-D region typing. The PLT technique takes advantage of the fact that peripheral blood lymphocytes can be primed to histocompatibility antigens in tissue culture in a way that commits these primed cells to respond in a rapid (second set) fashion when re-exposed in tissue culture to these same stimulating specificities. As the secondary (typing) phase of the PLT can be completed within 24 hours, it has great clinical potential. In addition to the potential for use as a rapid test, lymphocytes can be primed to a wide variety of specificities. Combinations of primary phase responding and stimulating cells can be co-cultured to select for nearly any HLA-D region antigen thus permitting the rapid identification of new specificities.

Using selective priming PLT techniques this laboratory was the first to split HLA-D and HLA-DR into two distinct activities. That is, HLA-D and HLA-DR specificities can be encoded by two separate sixth chromosome genes. This was demonstrated in a family where one histocompatibility complex haplotype of a parent clearly serotyped as HLA-DRw2 but clearly was not HLA-Dw2 by HTC typing. It was shown that primed lymphocytes could be generated to the DRw2 specificity alone and other primed lymphocytes generated to the HLA-D (non-Dw2) specificity (paper enclosed). Further, this was the first laboratory to use this PLT technique to confirm the identity of a new HLA-DR sero specificity. HLA-DRw4x7 was found to segregate in a family. Primed cells were generated to this specificity which reacted with all family members and panel members who carried the new specificity (paper enclosed).

In spite of the great potential for PLT testing, one hurdle to its wide spread use is a standardized technical and analytical approach to this data. In the secondary test phase of the PLT test the major problem is the wide fluctuation in the technical capacity of secondary stimulator cells to function. That is, a cell which is a poor stimulator cell frequently fails to stimulate a lymphocyte primed to a shared specificity. This laboratory has developed a new technique which standardizes the stimulating capacity of this cell and this allows uniform results. In this standardization technique, primed cells are stimulated with several concentrations of secondary phase stimulating cells. The CPM of the secondary response is then calibrated to the response of the stimulator cells to a B cell mitogen (poke weed mitogen).

Computer models are being developed which will permit a rapid and precise identification of the genetics of the cell being tested.

## 2.2 Expansion of Primed Cells

As the PLT test becomes possible for clinical transplantation testing, it will become important to make large numbers of these primed cells available. The initial generation of primed cells in the primary phase of the PLT produces a number of primed cells approximately equal to the number of initial unprimed responding cells. That is, where  $50 \times 10^6$  responding cells and  $50 \times 10^6$  stimulating cells are co-cultured in the primary phase, approximately  $50 \times 10^6$  primed cells are recovered for use in the secondary phase. Current techniques require  $75 \times 10^3$  primed cells for each secondary phase test. Approximately 750 tests can be obtained for each  $50 \times 10^6$  initial responding cells.

In order to expand this number of primed cells, a simple technique using conditioned media was employed. Conditioned media (CM) is a supernatant from lymphocytes grown for 24 hours in phytohemagglutinin. Lymphoid cells which have been previously stimulated will continue to replicate in tissue culture for indefinite periods in the presence of conditioned media. We have been able to expand the number of primed cells 50 fold after 14 day treatment with CM without loss of specificity (see enclosed preprint). Thus 37,500 tests can be obtained from  $50 \times 10^6$  initial primed cells.

In addition to expanding the number of primed cells available for typing, CM can be used to expand clones of primed cells. Primed cells of the PLT are in fact primed to a wide variety of cell surface antigens. This heterogeneity of the primed cells is due to wide range of affinities of antigenic determinants and the large



number of different stimulating antigens present on the cell surface. However any one cell is capable of responding to an extremely restricted range of specificities. Using CM, single cells can be permitted to replicate in tissue culture. These clonal PLT cells will produce much more restricted secondary (typing) responses. Thus cloned primed cells should be useful as highly specific test reagents.

A technique for cloning primed cells has been developed at the Georgetown facility. Blast cells are harvested after six days in primary phase culture. The cells are diluted so that each 0.1 ml contains one cell capable of replicating. An equal volume of conditioned media is added and the cells distributed in a 96 well tissue culture plate at 0.2 ml per well. When visible colonies are seen in the tissue culture wells they are transferred to progressively larger tissue culture vessels until adequate numbers of cells have replicated to permit testing.

### 3. Advances in Parallel Research Areas

#### 3.1 Separation of Functional Memory Cells

Characterization of the subtype of cell involved in the PLT, the memory cell, is of potential importance as this may lead to methods of blocking in vivo secondary reactions leading to transplant rejection. For example, it may become possible to develop an antiserum which could specifically prevent memory cells from functioning in transplant rejections. In these studies a rabbit antiserum specific for human T lymphocytes was used to bind to primed cells. The antiserum treated primed cells were then treated with a fluoresceinated goat anti-rabbit antibody. Cells brightly



stained were those cells with a high density of antibody from anti-human T antiserum. These fluorocinated cells were then passed through the fluorescence activated cell sorter, and divided into low, mid and high fluorescence cell pools. These pools were then tested in a secondary phase of the PLT. The great majority of the active cells in the secondary phase were the cells with a high density of bound anti-human antiserum (abstract enclosed).

### 3.2 Genetics of Circulating Cytotoxic Lymphocytes

It has been observed that there is a large person to person variation in the number of circulating cytotoxic lymphocytes (called natural killer cells). It has been hypothesized that the frequency of these circulating killer cells may be associated with certain disease states. To test if the level of natural killer cells was related to major histocompatibility complex genotype, panel members and homozygous typing cells were tested for their ability to kill chromium labeled target cells. It was determined that HLA-Dw3 and 4 were associated with high natural killer cell activity (abstract enclosed).

### 3.3 Use of PLT to Test Use of Interferon in Transplantation

Interferon is a material which can be produced by lymphocytes in response to viral infection. It is hypothesized that this material prevents the spread of viral infection. The secondary phase of the PLT was used to test whether interferon acts on memory cells. It was found that reactivity in the secondary phase of the PLT could be reduced by 50% with interferon. Thus it is possible this material may be used as a reagent in transplantation to prevent viral morbidity, and as an immunosuppressant.

### 3.4 Transformation of Homozygous Typing Cells into B Cell Lines

HLA-DR reagents are identified by their cytotoxicity to B cells. As most lymphoblastoid cell lines are in fact continuously growing B cells, they are a valuable source of reagent to act as a standard to test serologic reagents. As homozygous typing cells have restricted complexity of their major histocompatibility complex, lymphoblastoid cell lines of these HTC are ideal reagents to act as standards against which B cell antisera can be tested and defined. HTC's from this laboratory have been used extensively in both Navy programs and programs at other institutions for the refinement of B-cell serologic typing.

#### F. PROPOSAL FOR CONTINUATION OF THIS CONTRACT: WORK PLAN

The success of this contract to date has been in a number of areas: (1) developing technical facilities and expertise; (2) developing internationally accepted reagents; (3) expanding the knowledge of HLA-D region genetics; (4) using the developed reagents and expertise in local HLA-D typing; (5) support of other Navy and contract facilities, and (6) use of these reagents to better understand the fundamental role of this genetic region on transplantation immunology. We propose to continue developing along these established lines.

A) Identification of additional HLA-D region specificities: To date, eleven HLA-D region specificities have been accepted by the World Health Organization Committee on nomenclature. Work from the ongoing Eighth International Histocompatibility Workshop

should result in the addition of three to six new specificities in February of 1980. The extensive involvement of this laboratory in this workshop will contribute to this expansion of defined specificities and will enable the identification of local reagents for these specificities. These new typing reagents will be both homozygous typing cells and primed lymphocytes. These reagents will be made available to the Navy Transplantation program for prospective typing of both kidney and bone marrow transplant recipients and donors.

B) Expansion of understanding of HLA-D region genetics: Eighth International Histocompatibility Workshop work will identify new HLA-D and DR specificities, identify the physical chromosomal relationship of the major histocompatibility complex (MHC) components and the relationship of the MHC to other nearby genes such as the genes controlling several complement components.

C) Primed lymphocyte investigations: We propose to continue studying the relationship of the HLA-D region genes using primed lymphocytes. We are planning a Second International Workshop on Primed Lymphocyte Typing for April, 1980. All major international investigators in the PLT research area will be invited to a meeting to be held here in Washington to discuss advances in PLT technique, genetics and the immunology of these memory cells.

D) Production of large amounts of PLT reagents: We plan to extend the use of conditioned media to expand quantitatively the number of primed lymphocytes and make these reagents available to the Navy Transplant program.

E) PLT cloning techniques:

We plan to extend our primed lymphocyte cloning techniques. Cloned lymphocytes will become an extraordinarily valuable tool, magnifying our understanding of HLA-D region antigens and permitting us to develop exquisitely specific reagents. Furthermore, these clone techniques may represent the forerunner of an extraordinarily powerful clinical tool. Using these cloning techniques it may become possible to obtain a small number of peripheral blood lymphocytes from a histocompatible individual, treat these cells in vitro to obtain or deplete specific clones, expand the number of cells and then clinically transplant this material to patients with aplastic anemia resulting in the reconstitution of bone marrow function.

G. PUBLICATIONS AND PRESENTATIONS

During the past year five papers have been accepted for publication, four abstracts have been submitted and five presentations given.

Publications:

1. Pappas, F., Bonnard, G.D., Hartzman, R.J. and Strong, D.M., Human PL Cells Grown as Continued T Cell Lines, Transplantation Proceedings, (in press).
2. Hartzman, R.J., Amos, D.B., Pappas, F., Johnson, A.H., Ward, F., Romano, P.J., and Sell, K.W., Specificity of Primed LD Typing: The Major Reactions, Transplantation Proceedings, 9, p. 690, March, 1979.



3. Hartzman, R.J., Summary of the First International Workshop on Human Primed LD Typing, Tissue Antigens, (in press).
4. Johnson, A.H., Pappas, F., Ward, F.E., Amos, D.B., and Hartzman, R.J., DuB 15: A B-Cell Specificity in the HLA-D Region, Transplantation Proceedings 10, p. 805, Dec. 1978.
5. Hartzman, R.J., Pappas, F., Romano, P.J., Johnson, A.H., Ward, F.E. and Amos, D.B., Dissociation of HLA-D and HLA-DR Using Primed LD Typing, Transplantation Proceedings 10, p. 809, Dec. 1978.

#### Abstracts:

1. Romano, P.J., Jaffe, A. and Woody, J.N., HLA Restriction of Human Natural Killing Activity. Leukocyte Culture Conference, 1979.
2. Hartzman, R., Ahmed, A., Pappas, F., Habbersett, R., Strong, M. and Woody, J., Cell Surface Characteristics of Memory Lymphocytes American Association for Clinical Histocompatibility Testing, 1979.
3. Pappas, F., Bonnard, G.D., Strong, D.M., Hartzman, R.J., Schendel, D.J., and Wank, R., Human PLT Cells Grown as Continued T Cell Cultures. American Association for Clinical Histocompatibility Testing, 1979.
4. Hartzman, R.J., Amos, D.B., Johnson, A., Ward, F., Pappas, F., Romano, P. and Sell, K.W., The Genetic Complexity of Primed LD Typing, Transplantation Congress, 1978.

#### Presentations:

1. Pappas, F., Expansion of Primed Lymphocytes with Conditioned Media, American Association for Clinical Histocompatibility Testing, San Diego, California, April, 1979.

2. Ayres, J., Identification and Utilization of Homozygous Typing Cells, American Association for Clinical Histocompatibility Testing, San Diego, California, April, 1979.
3. Romano, P., HLA Restriction of Human Natural Killer Activity, Leukocyte Culture Conference, Ottawa, Canada, April, 1979.
4. Pappas, F., Development of Primed Lymphocyte Typing, American Association for Clinical Histocompatibility Testing, Boston, Mass., June, 1978.
5. Ayres, J., HLA-D Testing Using Homozygous Typing Cells, American Association for Clinical Histocompatibility Testing, June, 1978.

HUMAN PL CELLS GROWN AS CONTINUED

T CELL CULTURES

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the naval service at large.

## INTRODUCTION

The antigens responsible for the mixed lymphocyte reaction (MLR) are controlled by the HLA-D locus of the major histocompatibility complex (1). Cells sensitized in MLR and cultured for ten days represent primed lymphocytes (PL) that undergo rapid proliferation to lymphocytes bearing the specific sensitizing HLA-D and DR antigens in a secondary MLR (the PLT test) (1,2,3). Thus, PL may be used as a rapid method to identify HLA-D region antigens. One major drawback of this technique has been the difficulty in obtaining sufficiently large numbers of PL with a given specificity. An alternate means of generating cellular reagents, active and specific in the PLT, was highly desirable.

Conditioned media (CM) from PHA-stimulated leukocytes have been used to maintain long-term cultures of human peripheral blood T cells (4,5). These cultured T cells (CTC) retain several functions of T lymphocytes and respond in mixed lymphocyte reactions (MLR).

## MATERIALS AND METHODS

Both commercial (Associated BioMedics) and CM prepared in the laboratory at NIH was used (6). RPMI-1640 with 10% fetal bovine serum (FBS), 10% CM, 50 ug/ml gentamycin, and 35mM L-glutamine was used in all experiments.

PL cells were prepared according to the technique of Sheehy et al (2). HLA-D homozygous typing cells (HTC) were used as responders and either HTC or HLA-D heterozygous cells as stimulators (7). On day 10, PL were either used fresh or cryopreserved according to the method of Strong et al (8). Frozen-thawed or fresh PL were cultured with CM as cultured T cells (PL-CTC) in 50ml tissue culture flasks (Falcon, Model



3013, Oxnard CA.) in concentrations of  $5 \times 10^5$  cells/ml. Cell counts using trypan blue exclusion to determine viability were performed every 2 days. During the culture period, cell concentrations were maintained at 1 to  $3 \times 10^5$ /ml. A portion of the replicating cells were removed at intervals between the seventh and thirty third day in CM. The cells were removed from CM, allowed to rest 24 hours in RPMI 1640 with 10% FCS and then frozen in 7.5% dimethyl sulfoxide using a control rate freezer.

To determine if cells retained specificity the frozen PL-CTCs and the original primed cells (not treated with CM) were restimulated in a secondary phase with a panel of HLA-D typed heterozygous and homozygous cells. Briefly,  $2.5 \times 10^4$  responders were co-cultured with  $5 \times 10^4$  stimulator cells in 96 well round bottom plates (LINBRO MRC 96 TC, Hamden, CT.) for 48 hours, labeled with 1  $\mu$ Ci of  $^3$ H-thymidine (2 Ci/mM) and harvested after an additional 12 hours using a MASH. Incorporation of thymidine was then measured.

## RESULTS

PL-CTCs developed from frozen PL cells. PL cells representing seven established specificities (DW1-DW7) previously generated and cryopreserved were thawed and cultured with CM over a period of one month. A total of 14 cells, two for each specificity, were tested.

Maximum yield from most of the CM treated cells was reached between 12 and 15 days in culture. Starting with  $2.5 \times 10^6$  cells, one primed lymphocyte (PL5) reached  $74.5 \times 10^6$  cells by 12 days of CM culture. days. This was the maximum seen using frozen PL cells. The average increase was 15 times the number of initially cultured cells. Some of the cells (PL10 and PL12) doubled in the first 5 days in culture but

then began to decrease. All but two of the frozen thawed cells (PL2, PL13) showed poor survival after 16 days in culture.

Table 1 and Table 2 show the results of six of these cells when restimulated with a panel in secondary phase. In Table 1, PL4 retained its discriminatory ability up to the 16th day. In fact this primed cell appeared more selective after expansion with CM. The two HLA-Dw4 panel members giving "false positive" responses with the non-expanded PL4 do not restimulate the 13 and 16 day cells. PL5 and PL6 however, lost most of their secondary phase reactivity after treatment with CM. In Table 2, primed lymphocytes 7, 8 and 11 demonstrate generally equal or greater selectivity of the CM treated cells.

PL-CTCs developed from fresh PLs. These cells were placed in CM directly from their 10 day primary phase. Starting with  $2.5 \times 10^6$  cells, two of the cells had reached  $45 \times 10^6$  and the third  $32 \times 10^6$  by the seventh day in culture. Some of the cells were frozen and others allowed to continue. Unfortunately, the CM being used up to this point was switched to another lot that functioned poorly. It was decided to stop the cultures at day 22, since the cells began to decline in numbers.

The results of secondary phase stimulation of the unexpanded, day 7 and day 22 CM treated cultures are seen in Table 3. Again the treated cells besides maintaining their specificity show in some cases better discrimination between positives and negatives although the overall CPM is somewhat decreased.

#### DISCUSSION

Results from this study show that functional PL can be expanded using CM. These cells maintain specificity and in some cases even showed increased discrimination. However, several problems were encountered. In general the absolute CPM obtained from PL-CTC were

decreased although typing responses were easily detected. In the initial experiment using CTC generated from frozen-thawed PL, cell viability became a problem beyond 14 days in culture with CM. Additional experiments with fresh (day 10) PL-CTC suggested that this may have simply been a technical problem and that these cells can be grown in a manner comparable to that described for other CTC (5,6) with starting cultures of  $5 \times 10^6$  cells developing into  $2 \times 10^8$  cells within 14 days. Further experiments are planned to answer the more important question as to whether 16-30 day old PL-CTC lose their specificity.

Thus large numbers of PL primed for each of the HLA-D region specificities can be generated using the PL-CTC system. This will allow for a more practical application in the transplant situation, since adequate numbers of cells can be made available for typing more individuals. Further, the ability to maintain continued growth of primed cells in vitro will permit numerous other studies such as, characterization of the idiotypic nature and diversity of receptors for cloned PLT cells.

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Table 1. Secondary Phase Stimulation of PL-CTCs from Frozen PLs

Stimulator Panel	HLA-Dw Type	Responder Cells									
		PL 4		PL4-CTC		PL 6		PL6-CTC		PL 5	
		3a2*		DAY 13	DAY 16	4a3		DAY 13	DAY 16	4a3	PL5-CTC
											DAY 13 DAY 16
	4/4	16,908 <sup>†</sup>	1,274	479	618	302	178	716	-	-	127
	2/4	35,145	6,854	3,242	360	288	391	426	-	-	141
	3/3	390	114	182	20,556	933	119	21,219	-	-	85
	2/2	49,248	15,108	7,750	1,152	265	114	1,493	-	-	190
	4/4	14,342	448	- <sup>‡</sup>	196	98	84	516	126	84	
	3/3	2,256	137	-	36,974	1,490	721	44,713	716	149	
	3/3	3,087	181	156	36,724	1,931	739	29,998	753	337	
	2/2	48,142	12,067	6,261	1,441	192	299	2,881	-	133	
	2/4	38,640	8,167	4,044	1,618	353	264	1,655	-	170	

\* 3a2 represents HLA-Dw 3/3 homozygous cell primed to an HLA-Dw2 homozygous cell

† CPM

‡ NOT DONE

Table 2. Secondary Phase Stimulation of PL-CTCs from Frozen PLs

Stimulator Panel	Responder Cells									
	PL 7		PL7-CTC		PL 8		PL8-CTC		PL-11	
	HLA-Dw Type	2a4*	DAY 13	DAY 16	2a2/4	DAY 13	DAY 16	2a6	DAY 13	DAY 16
2/2		5,369 <sup>†</sup>	1,459	1,154	2,429	609	608	2,667	849	891
7/7		2,280	637	308	1,967	436	274	1,833	118	173
4/4		23,381	13,328	6,283	38,003	7,592	6,222	4,267	419	423
2/4		10,776	6,151	3,122	26,396	2,399	1,338	872	121	124
6/7		10,052	4,334	- <sup>‡</sup>	18,267	1,954	1,322	14,249	3,557	2,861
6/6		3,585	1,388	-	1,328	2,627	1,033	32,340	3,387	3,319
5/5		20,039	7,133	-	23,137	6,955	6,492	15,943	2,067	2,062
5/6		13,092	5,049	-	11,311	3,341	2,548	25,202	2,491	2,844
5/?		4,840	-	-	3,519	565	512	602	255	260
4/4		27,565	10,289	5,520	15,170	6,336	4,006	1,634	-	168
2/4		18,311	7,629	3,936	34,514	3,179	1,755	685	-	139
3/3		2,874	-	-	5,917	435	441	6,600	-	516
1/1		7,018	-	-	5,503	1,503	1,593	7,182	-	-
3/7		8,526	-	-	3,798	4,089	3,500	12,085	-	-
6/?		5,772	-	-	7,752	251	225	21,000	2,345	1,349

\* 2a4 represents HLA-Dw 2 homozygous cell primed to an HLA-Dw4 homozygous cell

† CPM

‡ NOT DONE

Table 3. Secondary Phase Stimulation of PL-CTCs from fresh PLs

Stimulator Panel	HLA-Dw Type	Responder Cells							
		PL 1		PL 2		PL 3		PL 3-CTC	
		2a1*	2a1*	2a7	2a7	3a4	3a4	DAY 7	DAY 22
		DAY 7	DAY 22	DAY 7	DAY 22	DAY 7	DAY 22	DAY 7	DAY 22
2/2	2,724 <sup>†</sup>	649	1,070	4,315	2,698	11,962	3,447	3,875	
3/3	7,697	3,184	1,212	5,498	2,827	1,687	375	878	
1/1	24,622	16,984	4,569	3,271	2,982	3,821	1,643	2,163	
7/7	2,680	1,338	918	14,605	8,113	4,510	1,433	1,602	
4/4	13,217	7,224	6,341	9,464	2,982	32,658	19,359	10,193	
1/3	18,220	9,387	3,278	2,193	989	3,306	859	415	
1/4	9,985	4,740	4,387	1,544	2,820	2,776	3,097	2,514	
1/8	30,631	23,138	11,114	7,324	5,567	13,610	5,605	6,685	
5/7	11,622	3,614	4,680	19,435	11,497	13,037	4,046	5,353	
3/7	6,978	3,868	1,457	15,554	9,705	6,900	2,448	2,343	
7/?	8,605	2,370	2,043	22,198	8,329	13,229	5,882	4,047	
3/7	10,147	4,382	2,163	21,530	13,324	6,290	2,121	2,680	
2/4	5,810	1,471	964	3,683	1,186	11,585	5,686	2,979	
2/?	2,132	971	1,000	2,828	2,704	6,584	1,191	2,279	
4/5	12,756	7,919	6,891	13,389	10,455	20,064	11,651	8,437	

\* 2a1 represents HLA-Dw2 homozygous cell primed to an HLA-Dw1 homozygous cell

† CPM



## Specificity of Primed LD Typing: The Major Reactions

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**H**UMAN lymphoid cells are capable of expressing a large number of genetically determined cell surface characteristics. The most extensively studied of these characteristics are the polymorphisms, which are controlled by a small segment of the sixth chromosome in man called the major histocompatibility complex (MHC). To date five distinct genetic regions, or loci, have been defined within the MHC: *HLA-A*, *B*, *C*, *D*, and *DR*. The identification of the first three of these has evolved over the past 20 years primarily through the efforts of many investigators studying complement-dependent cytotoxic activity of antisera, usually generated by maternal sensitization to paternal antigens during pregnancy or by planned immunizations.

Recently two additional loci have been localized to the MHC. These two loci, *HLA-D* and the closely associated *HLA-DR*, are of particular interest because they may represent homologues of genes in the mouse *I* region that are closely associated with control of certain immune functions. Thus our understanding of these histocompatibility loci may lead to the advancement of knowledge of immunologic regulation, disease development, and transplantation reactions.

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*HLA-D* typing is defined by the mixed leukocyte reaction (MLR). Although the antigens that stimulate MLR were initially thought to be controlled by the *HLA-A* and *B* loci, studies of recombinant families demonstrated that the primary determinants of MLR activation were located in at least one separate genetic locus within the MHC.<sup>1</sup> Individuals who possess a particular *HLA-D* specificity respond at a relatively low level in MLR to cells from individuals who are homozygous for a same *HLA-D* determinant. Selected homozygous cells used for typing purposes are called homozygous typing cells (HTC).<sup>2-4</sup>

The other recently demonstrated *HLA* polymorphism (*HLA-DR*) is defined by serologic reactions with peripheral blood B lymphocytes.<sup>5,6</sup> These serologic reactions were initially categorized on the basis of their association to *HLA-D* as defined by the HTC method. Although population studies demonstrated a very high correlation between *HLA-D* and *HLA-DR* specificities, numerous individuals who possess a particular *HLA-DR* specificity without the associated *HLA-D* type have been identified in recent studies of individuals and families. Conversely, a few individuals with a particular *HLA-D* specificity typed for an unassociated *DR* type.

As HTC and B cell typing progressed, a third new system of testing for cell surface determinants emerged.<sup>7</sup> This new system, called primed LD typing (PLT), is based on the observation in mouse and man that lymphocytes that have been activated in vitro can undergo a second set response when reexposed to the specific sensitizing antigens.<sup>8,9</sup> Initially these specificities defined by PLT (called PL specificities) were identified in families outside the context of *HLA-D* or *DR* typing.

It was clear from the outset that PLT described an exceptionally complex set of

determinants.<sup>10</sup> Only one-third of primed lymphocytes generated by intrafamilial priming demonstrated MHC associated specificity; some primed cells gave secondary restimulation patterns both within the family and with unrelated panels, which clearly indicated MHC antigens as the primary determinants. However, many primed cells were restimulated by nearly all secondary-phase stimulator cells.

Initially, the generation of primed cells with MHC specificity was restricted to family members who shared one MHC haplotype. However, other methods of priming were soon introduced. For example, the use of HLA-D-defined cells in the system, and, in particular, the use of homozygous typing cells as primary-phase stimulators and responders greatly increased the frequency of generating highly specific primed cells.<sup>11-13</sup>

At first, correlations between specificities identified using PLT typing and HTC typing were shown to be very high, suggesting that PLT defined HLA-D; however, it was later demonstrated that the correlation between HLA-DR and PLT was greater than the correlation between PLT and HLA-D.<sup>14</sup> It is now thought that either HLA-DR<sup>15</sup> or HLA-D<sup>16</sup> can act as priming and restimulating antigens.

To further define the genetic bases of PLT typing, we performed studies using both family members and unrelated individuals extensively typed for their HLA-A, B, C, D, and DR specificities. One particularly informative family with an HLA-B/D recombinant child is discussed here.

#### MATERIALS AND METHODS

##### *Source of Cells*

Lymphocytes were purified from peripheral whole blood obtained from our HLA-A, B, C, D, and DR-typed reference panels. Antisera and homozygous typing cells were from the Seventh International Histocompatibility Workshop and from locally available antisera and typing cells.

##### *MLC and HTC Typing*

The MLC technique has been previously described.<sup>17,18</sup> Data were analyzed using the 75th percentile double normalized value (DNV) method.<sup>19</sup>

##### *The Primed LD Typing (PLT)*

The PLT method was essentially the technique described by Sheehy and Bach.<sup>20</sup> In the secondary phase  $2.5 \times 10^4$  untreated stimulator cells were incubated for 48 hr, labeled with  $1.2 \mu\text{Ci } ^3\text{H-thymidine}$  ( $2 \text{ Ci/mM}$ ), and harvested after 12 additional hours (method 1). In addition, some experiments were performed using a tissue culture method that permitted the development of a new normalization technique devised to compensate for the technical variability of the secondary-phase stimulator cells. In these experiments  $2.5 \times 10^4$  PLT cells and two concentrations of secondary-phase stimulator cells ( $5 \times 10^4$  and  $1 \times 10^5$ ) were incubated for 48 hr, labeled with  $1.2 \mu\text{Ci } ^3\text{H-thymidine}$  ( $5 \text{ Ci/mM}$ ), and harvested after 12 additional hours (method 2). The secondary-phase response counts were adjusted to correspond to an estimated number of stimulator cells capable of producing a standard count (cpm) in response to pokeweed mitogen.<sup>21</sup> The negative control cpm values were then subtracted and the results expressed as a percentage of the positive control values.

##### *HLA-DR Typing and Serum Adsorption Studies*

HLA-DR typing was performed using B cells positively selected on an anti-F(ab)<sub>2</sub> monolayer.<sup>22</sup> A modification of the standard Oxford Workshop technique, which employed a shorter incubation time with complement (60 min versus 120 min), was utilized. Antigen assignments were based on the Oxford analysis of specificities as well as a Duke computer program for assignment of local antigens. For adsorption studies, two sequential adsorptions were performed for each cell using  $7 \times 10^6$  Ficoll-Hypaque-separated lymphocytes for each adsorption per 100  $\mu\text{l}$  of antiserum for 15 min on ice. Unadsorbed and lymphocyte-adsorbed antisera were tested for residual activity against B lymphocytes from family members and against five individuals reactive with the unadsorbed antiserum. Reference DRw2 antiserum 7w017 was used for the adsorptions.

#### RESULTS

The HLA specificities of family 001 have been extensively studied (Table 1). The family was typed with Seventh International Workshop sera for the HLA-DR specificities on three occasions, each time in duplicate. Of particular interest, DRw2 was defined on each paternal haplotype by Oxford Workshop antisera 7w013, 016, 017, 007, 010, 015, 021, 060, 065, 066, 067, 088, 105, 114, 138, 141, 144, 156, 158, 162, and 179. In addition, cells from siblings 4 and 5 could adsorb the DRw2 antiserum 7w017 with equal facility. The

Table 1. HLA Specificities of Family 001

	Father				
	A2	C-	B5	Dw(A <sub>2</sub> )	DRw2
a*	A2	C-	B5	Dw(A <sub>2</sub> )	DRw2
b	A1	C-	B7	Dw2	DRw2
	Mother				
	Aw30	C-	Bw44	Dw7	DRw7
c	Aw30	C-	Bw44	Dw7	DRw7
d	Aw26	C-	Dw38	Dw(B <sub>2</sub> )	DRw 4 × 7
Sibling 1, a/c					
Sibling 2, recombinant a/cd:					
a	A2	C-	B5	Dw(A <sub>2</sub> )	DRw2
c/d	Aw30	C-	Bw44	Dw(B <sub>2</sub> )	DRw 4 × 7
Siblings 3 and 5, a/d					
Sibling 4, b/d					

\* Haplotype.

Oxford Workshop sera 7w056, 057, 038, and 115 which defined DuB15(wla4×7), typed the maternal *d* haplotype. This DuB15 specificity had a frequency of 0.025 on the Duke reference panel. Cells classified as the DuB15 specificity do not react with DRw7 antisera and demonstrate only occasional weak reactions (30%–40% cytotoxicity) with two reference DRw4 antisera (7w037 and 059).

The HLA-D specificities were also studied with repeat typings. Over 300 typings for Dw1–Dw11 were performed using Seventh International Workshop and locally available HTC. The *b* haplotype was clearly Dw2 (average DNV 21.6 for 18 determinations). Haplotype *c* was clearly Dw7 (average DNV 24.4 for 13 typings) with some crossreactivity to Dw11 (average DNV 47.8 for 12 typings). The paternal (A<sub>2</sub>) and maternal (B<sub>2</sub>) specificities on the *a* and *d* haplotypes, respectively, represent currently undefined HLA-D specificities. Of particular interest in light of the *a* haplotype carrying the DRw2 specificity, the average DNV for 26 typings of this haplotype with Dw2 HTC was 89. Also of interest, the *d* haplotype (HLA-A26, Bw38, DuB15) gave an average DNV of 71 for 6 typings with Dw10 HTC, an average DNV of 85 for 19 typings with Dw4 HTC, and an average DNV of 79 for 9 typings with Dw7 HTC.

Primed lymphocytes were generated using family members primed against each other or

Table 2. Secondary-Phase Reactions to Lymphocytes Primed to HLA-Dw7, Dw(B<sub>2</sub>), DRw7, and DuB15

Secondary-Phase Stimulators	Primed Lymphocytes for Specificities*				
	Dw7 + DRw7			Dw(B <sub>2</sub> ) + DuB15	
	PC 1	PC 2	PC 3	PC 4	PC 5
Father Dw(A <sub>2</sub> )/2DRw2/2	38*	17	12	4	21
Mother Dw7/(B <sub>2</sub> )DRw7/DuB15	148	100‡	100‡	112	100‡
Sib 1 Dw(A <sub>2</sub> )/7DRw2/7	142	81	78	41	0
Sib 2 Dw(A <sub>2</sub> )/(B <sub>2</sub> )DRw2/DuB15	94	0†	26	110	85
Sib 3 Dw(A <sub>2</sub> )/(B <sub>2</sub> )DRw2/DuB15	60	4	23	121	90
Sib 4 Dw2/(B <sub>2</sub> )DRw2/DuB15	69	13	0†	100‡	82
Sib 5 Dw(A <sub>2</sub> )/(B <sub>2</sub> )DRw2/DuB15	93	4	37	100	73
Unrelated panel members					
U1 Dw7/7DRw7/7	100‡	62	64	28	0†
U2 Dw3/7DRw3/7	66	87	80	NT	NT
U3 Dw3/7DRw3/7	99	88	91	47	4
U4 Dw2/7DRw2/7	108	82	92	NT	NT
U5 Dw2/4DRw2/4	37	20	7	28	4
U6 Dw2/4DRw2/4	NT	NT	NT	31	12
U7 Dw 2/2DRw2/2	0†	12	0	0†	7
U8 Dw 2/2DRw2/2	NT	NT	NT	NT	NT
cpm for positive control	3953	7606	10211	6455	7140

NT, not tested.

\*Percentage of positive control. Scoring for results: >70% of positive control, ++; 50%–70%, +; 25%–50%, ±; <25%, –.

† Negative control (primary-phase responder).

‡ Positive control (primary-phase stimulator).

Table 3. Secondary-Phase Reactions to Lymphocytes Primed to HLA-Dw2 and/or DRw2

Secondary-Phase Stimulators	Primed Lymphocytes for Specificities†				
	Dw2 + DRw2. PC 6	Dw2			
		PC 7	PC 8	PC 9	PC 10
Father*	104	100‡	100‡	100‡	100‡
Mother	0§	17	39	39	20
Sib 1	75	4	0§	24	5
Sib 2	31	0	11	8	0§
Sib 3	64	0§	26	19	4
Sib 4	100‡	80	79	90	84
Sib 5	20	0	7	0§	0
Unrelated panel members					
U1	NT	5	12	21	13
U2	NT	NT	NT	NT	NT
U3	NT	NT	NT	NT	NT
U4	115	105	92	96	98
U5	NT	NT	NT	NT	NT
U6	187	123	106	109	133
U7	163	137	127	122	129
U8	NT	134	102	150	127
cpm of positive control	51576	66785	83921	57805	83181

\* See Table 2 for specificities.

† Percentage of positive control.

‡ Positive control (primary-phase stimulator).

§ Negative control (primary-phase responder).

Table 4. Secondary-Phase Reactions to Lymphocytes Primed to Dw(A<sub>2</sub>) and DRw2

Secondary Phase Stimulators*	Primed Lymphocytes for Specificities†						
	Dw(A <sub>2</sub> )			Dw(A <sub>2</sub> ) + DRw2			
	PC 11	PC 12	PC 13	PC 14	PC 15	PC 16	PC 17
Father	100‡	100‡	100‡	258	90	160	203
Mother	27	44	45	0§	0§	0§	0§
Sib 1	78	89	85	282	100‡	93	134
Sib 2	77	76	103	173	55	100‡	83
Sib 3	66	91	82	274	93	92	100‡
Sib 4	5	9	0§	90	46	58	62
Sib 5	63	42	36	100‡	35	65	82
Unrelated panel members							
U1	11	11	15	NT	41	7	0
U2	NT	NT	NT	NT	NT	NT	NT
U3	NT	NT	NT	NT	NT	NT	NT
U4	20	24	33	179	75	90	43
U5	NT	NT	NT	NT	NT	NT	NT
U6	19	29	35	260	114	102	132
U7	2	0§	26	267	99	74	80
U8	0§	0	29	NT	119	81	55
cpm of positive control	56371	79137	45824	16619	50496	46791	31379

\* See Table 2 for specificities.

† Percentage of positive control.

‡ Positive control (primary-phase stimulator).

§ Negative control (primary-phase responder).



against homozygous typing cells. Tables 2-4 are summaries of the PLT results. PLT technical method 1 was used to obtain the results seen in Table 2 and method 2 used for the experiments shown in Tables 3 and 4. To differentiate the response of primed cells to Dw7DRw7 and Dw(B.)DuB15, primed cells were generated against the maternal HLA-D and DR specificities (Table 2). Primed cell (PC) 1 was generated against HLA-Dw7 and DRw7 in a Dw2 HTC responder and was restimulated by all cells with these specificities as well as cells with the Dw(B.)DuB15 specificities. The responder cells for PC 2 and 3 were in each case siblings who were Dw(B.)DuB15, which cells were primed to Dw7DRw7 and were restimulated with Dw7DRw7 but not with cells with the Dw(B.)DuB15 specificities. Two primed cells were generated against the haplotype carrying Dw(B.)DuB15, one (PC 4) with a Dw2 HTC primary phase responder and the other (PC 5) with a Dw7 HTC responder. Both PC 5 and 6 were specific for secondary restimulation by family members with the Dw(B.)DuB15 specificities.

In efforts to differentiate priming for Dw2 distinct from priming to DRw2, primed cells were generated to either Dw2 along with DRw2 or Dw2 alone (with the accompanying DRw2 specificity bypassed by using a DRw2-bearing responder cell) (Table 3). PC 7 was primed to both Dw2 and DRw2 and was restimulated by cells bearing Dw2 and DRw2 and was also stimulated by family members with the D(A.)DRw2 specificities. Responders used for PC 7-10, which were serologically typed as DRw2, were primed to Dw2. These PC were specific for family and panel members with the Dw2 specificity and were restimulated to a very low level by those family members with the paternal DRw2 without Dw2.

Table 4 demonstrates priming to the paternal *a* haplotype specificities. PC 11-13 were primed to the Dw(A.) specificity, with the associated DRw2 specificity bypassed using DRw2 responding cells in the priming phase.

These cells were restimulated only by family members bearing the Dw(A.) specificity. PC 15-18 were primed to both Dw(A.) and DRw2 and were restimulated by family and panel cells bearing either Dw(A.) or DRw2.

## DISCUSSION

Although PLT reactions are complex, a number of fundamental observations have been made in this series of studies using a unique recombinant family and a carefully selected panel of primed cells. First, the major specificity (or specificities) defined by this system is located within the HLA-D, DR region of the major histocompatibility complex. This localization is defined by the strength of the association of priming and restimulation with HLA-D and DR specificity, by the failure of either *HLA-A* or *B*-locus differences to stimulate (data not shown), and by segregation of primary and secondary stimulation with the *D* side of the *B-D* recombinant in this family.

Second, HLA-D differences in the absence of DR differences in two separate combinations were capable of generating and restimulating primed cells. This was shown by priming for Dw2 alone or for Dw(A.) alone using primary stimulators and responders both positive for DRw2. The range of reactivity was broader when priming was to both a DRw difference as well as to a Dw difference. This suggests (thirdly) that HLA-DR is also capable of priming and restimulating. Fourth, PLT can be used to identify D and/or DR specificities not currently defined by either HTC or B cell methodology.<sup>23</sup> Finally, the reactivity demonstrated by PLT is controlled by the genetically determined specificities of all three cells involved. A responding cell sharing one specificity with the primary-phase stimulator will not give a PLT to the same specificity on a second-phase stimulator. Thus the PLT represents memory cell induction and the second set stimulation determined by the specificities of the three interacting cells.

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## DuB 15: A B-Cell Specificity in the HLA-D, -DR Region

A. H. Johnson, F. Pappas, F. E. Ward, D. B. Amos, and R. J. Hartzman

**E**IGHT B-cell specificities (HLA-DRw1-7 and wla 8) were defined during the Seventh International Workshop. DRw1, DRw2, DRw3, and DRw7 were considered clearly defined. DRw4, DRw5, DRw6, and wla 8 were less well defined, and workshop DRw4 antisera appeared to contain "cross-reacting" antibodies to DRw5 or DRw7. The Oxford joint report stated that DRw4 could be well defined in the absence of DRw5 or DRw7. However, if the cell was positive for DRw5, then assignment for DRw4 should be based on reactivity of the wla4  $\times$  7 sera. If the cell was positive for DRw7, then antigen assignment for DRw4 should be based on the reactivity of DRw4 antisera.<sup>1</sup>

Analysis of our own population and family data has revealed that sera designated wla4  $\times$  7 by Oxford contain a distinct and unique specificity that we called DuB 15,<sup>2</sup> in addition to containing antibodies that react with all DRw4 and with most DRw7-positive cells.

### MATERIALS AND METHODS

#### Antisera

Antisera used were those in the Seventh International B-cell antiserum set. Those used by Oxford for definition of DRw4 (7w059, 7w129, 7w106, 7w037), DRw7 (7w051, 7w077, 7w069, 7w054, 7w052), and wla4  $\times$  7 (7w056, 7w057, 7w038, 7w115) were specifically considered.

#### Source of Cells

Lymphocytes were purified from peripheral whole blood obtained from our HLA-A, -B, -C, -D, -DR-typed reference panels. Sources of homozygous typing cells (HTC) used for B-cell typing were the following: Dw4—GYOD-V22 (Amos) and AWHI-V91 (Hartzman); Dw7—PBUR-V56-7w534 (Hsu); Dw11—ESMO-V27-7w533 (Hsu).

#### Assignment of B-Cell Antigens

Antigen assignments were based on the Oxford analysis of specificities as well as a Duke program for assignment of local antigens. Discrepancies between the two

computer assignments were resolved by inspection of the data sheets and repeat typing where indicated. The family was typed on three separate occasions.

#### B-Cell Typing

B cells positively selected on a Fab'2 monolayer were used for typing.<sup>3</sup> A modification of the standard workshop technique, which employed a shorter incubation time with complement (60 min versus 120 min), was utilized.

#### Primed LD Typing (PLT)

Primed lymphocyte typing was performed by the method of Sheehy et al.<sup>4</sup> with the following modifications: 25,000 PLT cells plus 50,000 secondary phase stimulator cells, labeled at 48 hr, and harvested at 60 hr. Results were analyzed by clustering the responses of each PLT cell into five groups. The approximate percentages of these five groups compared to the positive control are:  $\geq 90\%$  (+ +), 70%–90% (+ +), 50%–70% (+), 25%–50% ( $\pm$ ), and  $< 25\%$  (–). The highest two groups show definite specificity sharing, the middle group shows probable sharing, the fourth group demonstrates some cross-reactivity, and the last group is unreactive.

### RESULTS

#### Definition of DuB 15

The cytotoxic reaction patterns observed for DRw4, DRw7, or DuB 15 positive individuals, as well as the reaction patterns for Dw4, Dw7, and Dw11 HTCs, are given in Table 1. DRw4-positive cells react strongly with all

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Table 1. Antiserum Reactivity Patterns for DRw4, DRw7, and DuB 15

	DRw4				DuB 15 wla4x7				DRw7				
	059*	129	106	037	056	057	038	115	051	077	069	054	052
DRw4	+	+	+	+	+	+	+	+	-	-	-	-	-
DRw7	-	-	-	-	±	-	±	±	+	+	+	+	+
DuB 15	-	-	±	-	+	+	+	+	-	-	-	-	-
Dw4 HTC	+	+	+	+	+	+	+	+	-	-	-	-	-
Dw7 HTC	-	-	-	-	+	-	±	+	+	+	+	+	+
Dw11 HTC	-	-	+	-	+	-	±	+	+	+	+	+	+

\*Seventh International Workshop B-cell antiserum number.

wla4 × 7 sera. DRw7-positive cells react to varying strengths with 3 of the wla4 × 7 sera (7w056, 7w038, 7w115). Several unrelated individuals (2.5%) reacted strongly and reproducibly only with the wla4 × 7 antisera, giving, in addition, occasional weak reactions with two DRw4 antisera, 7w037 and 7w059. These cells have been called DuB 15.

One family with an HLA-B/D recombinant was informative for segregation of DuB 15. The recombinant family data are given in Table 2. DuB 15 segregates with the maternal d haplotype and independently from DRw7. The recombinant individual inherited the HLA-A and -B antigens from the maternal c and the HLA-D segment from the d chromosome and is DuB 15 positive.

#### Relationship to HLA-D

Primed lymphocyte test (PLT) cells were generated against the Dw-, DuB haplotype using appropriate family members as stimulating cells and homozygous typing cells (HTC) or family members as responders in the primary phase. Specific primed cells could be generated against the Dw-, DuB 15 haplotype. All family members positive for DuB 15, including the HLA-B/D recombinant, restimulated PLT cells made against the Dw-, DuB 15 haplotype (Table 2). Only the two unrelated individuals positive for DuB 15 (Dw3, DRw3, DuB 15 and Dw2, DRw2, DuB 15) restimulated these PLT cells to a level equal to restimulation by family

Table 2. Segregation of DuB 15 in an HLA-B/D Recombinant Family

		HLA-A	HLA-C	HLA-B	HLA-DR	HLA-D	DuB 15—PLT*
Father	a	1	—	7	w2	w2	—
	b	2	—	5	w2	—	—
Mother	c	w30	—	w44	w7	w7	++
	d	26	—	w38	DuB 15	—	—
Ch 1	b	2	—	5	w2	—	—
	c	w30	—	w44	w7	w7	++
Ch 2†	b	2	—	5	w2	—	++
	c/d	w30	—	w44	DuB 15	—	++
Ch 3	b	2	—	5	w2	—	++
	d	26	—	w38	DuB 15	—	++
Ch 4	a	1	—	7	w2	w2	++
	d	26	—	w38	DuB 15	—	++
Ch 5	b	2	—	5	w2	—	++
	d	26	—	w38	DuB 15	—	++

\*++ indicates restimulation in secondary phase ( $\geq 70\%$  of positive control) of a cell primed against the Dw-, DuB 15 haplotype in the primary phase. Two combinations were used in the primary phase: (1) R = Dw7 HTC, S = mother; and (2) R = Dw2 HTC, S = Ch4.

†HLA-B/D recombinant.



members. In addition, PLT cells were generated against Dw7, DRw7 in a Dw2, DRw2 HTC and in a Dw2, DRw2/Dw-, DuB 15 heterozygote family member. Two patterns of restimulation were observed. The PLT cell generated in the HTC had a broader pattern, being restimulated by DuB 15 positive cells as well as by Dw7, DRw7 positive cells. However, the PLT cell generated in a responder positive for DuB 15 was restimulated only by those individuals positive for Dw7, DRw7.

#### DISCUSSION

DuB 15 is a unique serologically defined specificity genetically different from DRw4 and DRw7. All individuals typing as DRw4

are positive with antisera reacting with DuB 2,22115. positive individuals may react weakly with DuB 15 antisera. However, DuB 15 positive individuals do not react with DRw4 or DRw7 antisera. The frequency of DuB 15 in our unrelated panel is 0.025. Individuals typing for DuB 15 have only one WHO-defined HLA-D and one HLA-DR specificity. A family with an HLA-B/D recombinant permitted mapping of DuB 15 to the left of HLA-B. Specific PLT cells could be generated against the Dw-, DuB 15 haplotype. PLT cells made against Dw7, DRw7 were restimulated by DuB 15 positive cells, but this reactivity was absent when the responder cell in the primary phase was positive for DuB 15.

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## Dissociation of HLA-D and HLA-DR Using Primed LD Typing

R. J. Hartzman, F. Pappas, P. J. Romano, A. H. Johnson, F. E. Ward, and D. B. Amos

**L**YMPHOID cells are capable of recognition of and subsequent activation by foreign histocompatibility antigens expressed by irradiated stimulating cells. This process of recognition and activation is demonstrated in the mixed leukocyte reaction (MLR). Although these antigens stimulating MLR were initially thought to be controlled by HLA-A and -B, studies with recombinant families demonstrated that the most potent determinant of MLC activation is within a separate genetic region called HLA-D located within the major histocompatibility complex (MHC).<sup>1,2</sup> Individuals homozygous for this major determinant were identified, and a system of using these lymphocytes as MLC typing reagents (called homozygous typing cells—HTC) as reference cells for HLA-D typing was developed.<sup>3-5</sup>

Shortly after the identification of the HLA-D region by HTC, a serologic method for identifying these specificities was sought.<sup>6,7</sup> Antisera that could differentiate MHC specificities on human B cells from those detectable on T cells were developed by screening, adsorption, and immunization procedures. The Seventh International Histocompatibility Workshop, as well as studies by individual laboratories, categorized B-cell typing antisera, which demonstrated patterns of reactivity that were highly associated with HLA-D type as defined by the HTC method. These HLA-D-associated specificities are now called HLA-DR (for D-related). However, certain individuals could be identified in whom one MHC haplotype was identified by a particular HLA-DR specificity but lacked the associated HLA-D type.

As these HTC and B-cell methods were being developed, a third typing method, called the primed LD typing (PLT) test, was devised.<sup>8</sup> The PLT method takes advantage of the fact that a lymphocyte activated in vitro

in MLR can revert to a memory cell and be specifically reactivated in a second-set fashion.<sup>9,10</sup> Initially, PLT typing laboratories reported high correlations between specificities defined by PLT and those defined by HTC typing.<sup>11</sup> As B-cell serotyping became available, it was reported that correlations between PLT typing and this serologic test were as great or greater than the correlations between PLT and HLA-D and that the major specificities defined by PLT might be the HLA-DR determinants.<sup>12</sup>

To define the roles of HLA-D and HLA-DR in primed LD typing, a family that had been extensively typed by both HTC and B-cell methods was studied using PLT techniques. This family was unusual as one paternal haplotype typed as HLA-Dw2, -DRw2 and the other haplotype as HLA-Dw(A\*), -DRw2. The (A\*) represents an HLA-D type that has not been defined by the HTC method. The use of cells from children, parents, and homozygous individuals (HTC) in the primary phase allowed the generation of primed cells against the HLA-Dw2 specificity

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with the HLA-DR effects bypassed or against the other haplotype carrying HLA-DRw2 [along with the undefined Dw(A<sub>\*</sub>)] without Dw2.

#### MATERIALS AND METHODS

##### *Mixed Leukocyte Culture (MLC) and Homozygous Typing Cell (HTC) Typing*

The MLC technique has been previously described.<sup>13,14</sup> Data was analyzed using the 75th percentile double normalization method.<sup>15</sup> A double normalized value of less than 40 was considered a typing response, 40–60 is unclear, and greater than 60 is a nontyping response.

##### *Primed LD Typing (PLT)*

The PLT method used was essentially the technique described by Sheehy et al.<sup>16</sup> Briefly,  $2.5 \times 10^4$  PLT cells and  $5 \times 10^4$  untreated stimulators were incubated for 48 hr, labeled with 1.2  $\mu$ Ci <sup>3</sup>H-thymidine (2 Ci/mM), and harvested after an additional 12 hr.

##### *HLA-DR Typing and Serum Adsorption Studies*

HLA-DR typing was performed using B cells positively selected on an Fab' monolayer.<sup>17</sup> Antigen assignments were based on the Oxford analysis of specificities as well as a Duke program for assignment of local antigens. For adsorption studies, two sequential adsorptions were performed for each cell using  $7 \times 10^6$  lymphocytes per 100  $\mu$ l of antiserum for 15 min on ice. Unadsorbed and lymphocyte-adsorbed antisera were tested for residual activity against B lymphocytes from family members and against five individuals reactive with the unadsorbed antiserum.

#### RESULTS

The HLA-A, -B, -C, -D, and -DR specificities of the family are listed in Table 1. "A star" (A<sub>\*</sub>) represents the undefined paternal, and "B star" (B<sub>\*</sub>) represents the undefined maternal HLA-D specificity. Four primed lymphocytes of differing specificities can be produced in this family by selecting different stimulator-responder combinations. For example the mother primed against sibling 4 stimulators provides cells primed to both DRw2 and/or Dw2, whereas sibling 1 cells responding to the father should be directed against Dw2, only as DRw2 is shared. Similarly, priming was carried out by stimulating a Dw7 HTC against sibling 4 to give an

Table 1. HLA Specificities of Family 001

	a*	A2	C-	B5	Dw(A <sub>*</sub> )	DRw2
Father:	b	A1	C-	B7	Dw2	DRw2
	c	Aw30	C-	Bw44	Dw7	DRw7
Mother:	d	Aw26	C-	Bw38	Dw(B <sub>*</sub> )	DRw4 x 7
Sibling 1:	a*/c					
Sibling 2:	Recombinant a/cd					
	a	A2	C-	B5	Dw(A <sub>*</sub> )	DRw2
	c/d	Aw30	C-	Bw44	Dw(B <sub>*</sub> )	DRw4 x 7
Siblings 3 and 5:	a/d					
Sibling 4:	b/d					

\*Haplotype.

anti-Dw(A<sub>\*</sub>) and/or DRw2 and a Dw2 HTC against the father to give anti-Dw(A<sub>\*</sub>).

The HLA specificities are well characterized in this family. In particular, Oxford Workshop antisera 7w013, 016, 017, 007, 010, 015, 021, 060, 065, 066, 067, 088, 105, 114, 138, 141, 144, 156, 158, 162, and 179 all react with each paternal haplotype. Cells from siblings 4 and 5 can adsorb DRw2 antiserum 7w017 with equal facility. Although the paternal haplotype is different in each of those two siblings, the adsorption removed all activity of this antiserum against cells from family members and five unrelated individuals against which this antiserum had been cytotoxic. The maternal cell was used as the negative control for adsorption and did not remove reactivity.

The HLA-Dw2 specificity was identified by repeated homozygous typing cell testing of each specificity. Eighteen determinations of reactivity of the father's cell against Dw2 HTC were made. The average double normalized value (DNV) of these determinations was 21.6 (SE  $\pm$  4.0). The HLA-D specificity of the other paternal haplotype was determined by testing cells from the four children who possessed the appropriate haplotype. Twenty-six determinations of reactivity of this second DRw2-carrying haplotype to stimulation by Dw2 HTC were made. The average DNV was 89 (SE  $\pm$  5.2). This non-Dw2 paternal haplotype was also tested

Table 2. Secondary Phase Reactions to Lymphocytes Primed to HLA-D or HLA-DR

Secondary Phase Stimulators	Primed Lymphocytes for Specificities				No PLT
	(1) Dw2 + DRw2	(2) Dw2	(3) Dw(A <sub>2</sub> ) + DRw2	(4) Dw(A <sub>2</sub> )	
Father	6,995 (++)*	2,642 (+c)	5,081 (++)	11,038 (+c)	746
Mother	0 (-c)†	779 (-)	3,703 (+)	0 (-)	595
Sib 1	3,171 (±)	0 (-c)	6,247 (+c)	11,173 (++)	1,152
Sib 2	4,891 (++)	63 (-)	6,381 (++)	10,988 (++)	1,827
Sib 3	4,037 (++)	0 (-)	3,923 (+)	10,309 (++)	697
Sib 4	6,597 (+c)‡	4,491 (++)	2,559 (±)	0 (-)	647
Sib 5	4,984 (++)	727 (-)	7,060 (++)	15,566 (++)	1,873
Unrelated a§	13,512 (++)	12,067 (++)	3,499 (+)	0 (-c)	1,606
Unrelated b§	9,558 (++)	10,163 (++)	2,289 (±)	0 (-)	1,254
Unrelated c§	11,568 (++)	7,983 (++)	3,429 (+)	0 (-)	2,226
None	2,170	1,848	659	1,795	—

\*Scoring for results: >70% of positive control (++); 50%-70% (+); 25%-50% (±); <25% (-). Numbers represent cpm of <sup>3</sup>H thymidine incorporation of cultures labeled at 48 hr and harvested 12 hr later, less PLT and stimulator cpm.

†(-c) is the negative control.

‡(+c) is the positive control.

§The HLA-D and -DR types of unrelated panel numbers shown are: (a) D 2,2, DR 2,2; (b) D 2,4 DR 2,4; and (c) D 2,7, DR 2,7.

against HTC representing the other known HLA-D specificities (Dw1-11) in 208 determinations. This haplotype was clearly not Dw1-11.

Primed lymphocytes were generated to differentiate the paternal specificities. Table 2 demonstrates the reactions of these primed cells against the family members plus the panel members possessing Dw2 and DRw2. Primed cell one is strongly restimulated by all panel members possessing both Dw2 and DRw2 and to a lesser extent by siblings who possessed the paternal DRw2 non-Dw2 specificity. Primed cell 2 (α Dw2) is specific for family and panel members with the Dw2 specificity. Of importance, the siblings with paternal DRw2, but not Dw2, produce low secondary stimulation. PLT cell 3 represents a Dw7 HTC primed to the Dw(A<sub>2</sub>) and DRw2. This cell is most strongly restimulated by cells that have both Dw(A<sub>2</sub>) and DRw2, but is also stimulated by cells with DRw2 and 5 of 12 other panel members (data not shown). Last, a Dw2 HTC was used as the primary phase responder and the father as the stimulator. This combination results in bypassing of the effects of both Dw2 and DRw2. The cell is strongly and specifically

restimulated only by cells possessing the new Dw(A<sub>2</sub>) specificity and not by cells with only the Dw2 or DRw2 specificity.

#### DISCUSSION

The data demonstrate conclusively that primed cells can be generated in vitro to non-HLA-DR specificities. Cells primed to Dw2 but not DRw2 could be restimulated with cells bearing Dw2 but not by cells bearing DRw2 without Dw2. Second, a new specificity associated with HLA-D(A<sub>2</sub>) could be generated independently of the DRw2 present on the same haplotype. Third, lymphocytes primed to DRw2 could be restimulated by cells bearing the DRw2 specificity independently of their Dw specificity. Last, cells primed to one HLA-D specificity, but a nonassociated HLA-DR specificity, could be restimulated with cells bearing either the appropriate D or DR specificity.

Thus, in two separate PLTs, HLA-D is capable of causing both induction of memory-cell production and second-set restimulation, and in those combinations in which there was also a DR difference, the reactivity was broadened, suggesting that DR could also prime and restimulate. The cells primed to



both, however, had rather complex restimulation patterns, especially on an expanded panel (not shown), and this reactivity is being further studied.

Although HLA-D and -DR appear to act as cell products, which cause both primary- and secondary-phase stimulation in the PLT test, it should be cautioned that other specificities associated with these types could be responsible. Thus, products of additional loci in the HLA-D (or DR) region could contribute to stimulation. However, at our current level of sophistication of D-region typing, it appears that both HLA-D and HLA-DR are separate loci that function as major and relatively independent factors in PLT typing.

The multiple specificities and loci interacting in PLT appear to be a major reason for

the relatively low frequency (30%–40%) of discriminant primed cells generated by intra-familial priming. The presence of these multiple specificities points to the necessity of characterizing the primary-phase responder and stimulator cells carefully for HLA-D and -DR before the results from these primed cells can be fully evaluated. However, the ability to generate cells against both D and the associated DR can be very useful for screening procedures and may be of great clinical significance.

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